7-3. Spore and sporulation

Daisuke Imamura* and Kazuhito Watabe
Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573-0101, Japan
*E-mail: imadaisuke@gmail.com

Abstract. The soil bacterium Bacillus subtilis must cope with the environmental change including availability of nutrients, temperature, salinity, pH, antibiotics, toxins, bacteriophage, radiation, and other factors capable of damaging cells. Therefore, B. subtilis cells are equipped with a genetically regulated program to adapt to these changes. Recently, several instances of multicellular behavior of bacteria to maximize their survival are revealed. Spore formation is the ultimate surviving strategy for longtime, which is distinct physiological state from vegetative cells. Sporulation involves a highly ordered program of gene expression and morphological change. Intercellular signal transduction and compartment-specific gene expression brings about maturation of the spore. Mature spores remain for long periods of starvation and are resistant to several environmental insults. These properties are attributed mainly to the unique figures of spore’s outer layers, spore coat and cortex, as well as to the physical state of spore cytoplasm. In this chapter, we describe about surviving strategies, regulation of spatiotemporal gene expression during sporulation, and structure and resistance of the spores of B. subtilis.
Introduction

Since the Gram-positive bacterium *Bacillus subtilis* inhabits soil, it must cope with the environmental change including availability of nutrients, temperature, salinity, pH, antibiotics, toxins, bacteriophage, radiation, and other factors capable of damaging cells. Therefore, *B. subtilis* cells are equipped with a genetically regulated program to adapt to these changes. Housekeeping \( \sigma \) factor, \( \sigma^A \), is a major \( \sigma \) subunit of RNA polymerase in exponentially growing *B. subtilis*. However, several stresses including energy stresses, such as those caused by carbon, phosphorus, or oxygen starvation, and environmental stresses, such as acid, ethanol, heat, or salt stresses induce activation of alternative \( \sigma \) factor \( \sigma^B \), which governs general stress response of cells [1, 2, 3]. General stress response by \( \sigma^B \) increases resistance of cells to multiple stresses [4, 5]. Traditionally, genetically identical bacterial cells in liquid media in well-stirred flasks were assumed to be identical in terms of gene expression and the pattern of protein synthesis, with some fluctuations. Therefore, microbiologists have monitored bacterial cell states using an average gene expression level. However, recent studies using fluorescence microscopy and flow cytometry revealed that genetically identical bacterial population in identical culture condition exhibits distinct physiological states without any mutation or DNA rearrangement [6, 7]. These heterogeneous behaviors maximize their chance of survival. On the other hand, ultimate long term surviving strategy of some Gram-positive bacteria is formation of endospore (spore). Spores are entirely distinct in morphology from vegetative cells and are highly resistant to several conditions including extreme temperature, low-nutrient environments, and harsh chemical treatment for over decades, centuries, or sometimes several million years. Special characteristics and peculiar morphology of spore confer such extreme dormancy. Here, we review surviving strategies of *B. subtilis*, spore formation, and structure and resistance of spores.

1. Heterogeneous behavior of *Bacillus subtilis*

Recently, several instance of multicellular behavior termed as biostability of *B. subtilis* to maximize their chance of survival are revealed. During the stationary phase, *B. subtilis* enter a physiological state known as genetic competence. *B. subtilis* cells in a state of competence can uptake DNA from the environment and incorporate the DNA into their genome by recombination [8]. The development of competence is an advantageous strategy to genetically adapt to a changing environment if it confers a beneficial new trait to the recipient. *B. subtilis* cells become competent
naturally as a culture enters stationary phase, but it occurs only in 10% to 20% cells in the population [9, 10, 11]. The alternative sigma factor $\sigma^b$ directs the transcription of genes involved in flagellum biosynthesis and motility [12, 13]. Kearns and Losick discovered that growing $B.\ subtilis$ cells are a mixed population of single swimming cells and chains of sessile cells [14]. Another example of biostability in $B.\ subtilis$ is sporulation, a developmental process that culminates in the formation of a dormant spore that is able to resist extreme environments. The sporulation process and resistance of spore are reviewed below in more detail. Briefly, sporulation is initiated in response to nutrient limitation and the master regulator for spore formation is Spo0A [15]. However, only some cells in a population activate Spo0A (spotulating cells) and some do not (non-sporulating cells) [16, 17]. Sporulating cells of $B.\ subtilis$ exhibit a behavior referred to as cannibalism in which the sporulating cells trigger the lysis of non-sporulating cells using a killing factor and a toxin [17]. Nutrients released from the lysed non-sporulating cells delay sporulating cells from becoming committed to spore formation. Spore formation is an elaborate process and it needs several hours (at least 6 hours to become heat resistant and 24 hours for majority to become mature spore). After the commitment to sporulation, sporulating cells cannot resume growth even if nutrients become available until the maturation of spore. Thus, it seems to be advantageous for $B.\ subtilis$ population to delay commitment of sporulation as long as possible by the cannibalism. Since sporulating cells do not lyse all non-sporulating cells, the culture of sporulating $B.\ subtilis$ are a mixed population of sporulating and non-sporulating cells. These heterogeneous behaviors of clonal population are surviving strategy of bacterium. Cells cannot know the future environment and therefore cannot determine which physiological states including growth, swimming, competence, stationary, and spore formation will be advantageous to survive. If all cells in the population become one state and failed, cells are threatened with extinction. Therefore, bacterial cells in clonal population hedge on several physiological states by heterogeneous behaviors to maximize their chance of survival.

2. Sporulation and gene regulation

Ultimate long term surviving strategy of some Gram-positive bacteria is formation of endospore (spore). Nutrient limitation is a major signal for initiation of sporulation in Bacilli and Clostridia. Spore formation of $B.\ subtilis$ is an elaborate differentiation process that involves a highly ordered program of gene expression and morphological change (Fig. 1)[18, 19]. A housekeeping $\sigma$ factor $\sigma^A$ is a major $\sigma$ factor in growing $B.\ subtilis$ cells.
Figure 1. Sporulation process and sigma cascade of B. subtilis. (a) Predvisional cell. σ^H and Spo0A become active in addition to σ^A at the initiation of sporulation. (b) Asymmetric division. Polar septum divides the cell into larger mother cell and smaller forespore. σ^F and σ^K become active only in the forespore and the mother cell, respectively. (c) Engulfment. Mother cell membrane migrates around the forespore membrane during a phagocytic-like process. (d) The completion of engulfment involves fusion of the mother cell membrane to pinch off the forespore within the mother cell, triggering activation of σ^G and σ^K in the forespore and the mother cell, respectively. (e) The cortex, a thick layer of peptidoglycan, is formed between the inner and outer membranes of the forespore. (f) Spore coat and crust proteins are produced in the mother cell and assemble around the forespore. (g) Mature spore is released upon lysis of the mother cell. Dashed arrows indicate intercellular signal transduction.
However, when cells encounter nutrient deprivation, sensor kinases, KinA, KinB, KinC, KinD, and/or KinE, undergoes autophosphorylation and trigger phosphotransfer reaction, termed phosphorelay [15, 20, 21]. Phosphate is transferred to a master transcriptional regulator Spo0A via two phosphotransferases, Spo0F and Spo0B [22]. Phosphorylated Spo0A (Spo0A~P) becomes a positive and negative transcriptional regulator for sporulation genes including abrB [23]. AbrB is a transcriptional regulator and represses a gene encoding alternative σ factor σ^H. Spo0A~P represses abrB thus leading to the stimulation of σ^H expression [24]. Spo0A~P regulates transcription of genes required during initiation of sporulation including genes for asymmetric septum formation by both RNA polymerase containing σ^A and σ^H (Fig. 1a and b). Genes transcribed by σ^A RNA polymerase and σ^F RNA polymerase include spoIIG operon and spoIIA operon encoding σ^E and σ^F, respectively, in the predivisional cell (Fig. 1a). σ^E is synthesized as an inactive precursor, pro-σ^E, and σ^F activity is repressed by SpoIIAB, an anti σ factor for σ^F. Therefore both σ^E and σ^F are kept inactive until the asymmetric division. Just after polar septation, σ^F becomes active by release from SpoIIAB only in the forespore (Fig. 1b) [25, 26]. This subunit of RNA polymerase directs transcription of 47 genes in the forespore including sigG and spoIIR [27, 28]. sigG encodes the next σ factor in the forespore, σ^G, however, it is inactive until the completion of engulfment (Fig. 1c and d). SpoIIR is believed to be secreted from the forespore into the space between the two membranes of the polar septum [29] (Fig. 1b). SpoIIR activates SpoIIGA, a signal transducing aspartic protease, on mother cell membrane [30, 31, 32, 33, 34]. SpoIIGA cleaves N-terminal 27 residues of pro-σ^E result in the formation of active σ^E only in the mother cell. σ^E RNA polymerase transcribes over 270 genes in the mother cell including a gene for σ^K [27, 35, 36, 37]. However, σ^K is synthesized as an inactive precursor, pro-σ^K, and it is inactive until mother cell receives a signal of σ^G activation from the forespore. The products of certain genes under σ^F control in the forespore and other genes under σ^E control in the mother cell bring about further morphological change (Fig. 1c and d). The mother cell membrane migrates around the forespore membrane during a phagocytic-like process called engulfment. The completion of engulfment involves fusion of the mother cell membrane to pinch off the forespore within the mother cell, triggering activation of σ^G in the forespore (Fig. 1d). σ^G RNA polymerase newly activates 81 genes in the forespore including spoIVB [28]. SpoIVB is believed to be secreted across the inner forespore membrane (former forespore membrane) into the space between the two membranes surrounding the forespore (Fig. 1d) and target to a complex of proteins including SpoIVFA, SpoIVFB, and BofA located in the outer forespore membrane.
(former mother cell membrane) [38, 39]. All three proteins in the complex are expressed in the mother cell under the control of $\sigma^C$ RNA polymerase [40, 41]. SpoIVB relieves BofA-mediated inhibition of SpoIVFB [42], a signal transducing metalloprotease, then SpoIVFB processes pro-$\sigma^K$ to active $\sigma^K$ in the mother cell [43]. $\sigma^K$ RNA polymerase activates 75 additional genes in the mother cell [36]. The products of genes under the control of $\sigma^D$ and $\sigma^K$ bring about maturation of the spore. The cortex, a thick layer of peptidoglycan, is deposited between the inner and outer membranes of the forespore and is responsible for maintaining the highly dehydrated state of the core, thereby contributing to the extreme dormancy and heat resistance of spores (Fig. 1e). Spore coat assembly involves the deposition of at least 70 protein species forming a proteinaceous shell [44, 45, 46] (Fig. 1f). These layers provide a protective barrier against bactericidal enzymes and chemicals such as lysozyme and organic solvents [47]. Mature spore is released upon lysis of the mother cell into the environment (Fig. 1g).

Spores are entirely distinct in morphology from vegetative cells and are highly resistant to several conditions including extreme temperature, low-nutrient environments, and harsh chemical treatment for over decades or centuries. One of the oldest spore-forming bacterium was isolated from a brine inclusion within a 250 million year-old salt crystal in the Permian Salado Formation as a revival form of a *Bacillus* species [48], and the other was found as a closely related to extent *B. sphaericus* in the 25-40 million-year-old Dominican amber [49]. Why do the bacterial spores show so high dormancy and resistant against abnormal condition. The answer will come from their characteristic nature and peculiar morphology.

### 3. Dormancy

Some Gram-positive bacteria of *Bacilli* and *Clostridia* form spores in response to change of environmental conditions such as nutrient deprivation. Bacterial spores have unique features, i.e. remain viable during long periods of starvation and are resistant to heat, toxic chemicals, lytic enzymes, and other factors capable of damaging vegetative cells [47]. The resistance of *B. subtilis* spore is summarized in Table. Although spores can remain dormant for several years, specific nutrients such as L-alanine and D-glucose can trigger the rapid ‘return to life’ through germination followed by outgrowth [51]. Because of having high resistance, serious concern is paid on food-born disease, medical supplies, and food industry for prevention of the pollution by spore-forming bacteria. Recently more interest has grown in the actual use of *B. anthracis* spores as a bioterrorism weapon.
The first step in sporulation is an asymmetric division of the cytoplasm, generating a larger mother cell and a smaller forespore (Fig. 1b). Second morphological change is migration of mother cell membrane around the forespore membrane during a phagocytosis-like process called engulfment (Fig. 1c and d) [52]. Progression of forespore development involves a series of events, including engulfment of the forespore by the mother cell plasma membrane, spore-specific layer structure such as germ cell wall and cortex, covering of the outer forespore surface with a complex proteinaceous coat, and in some species an outermost balloon-like layer termed exosporium. The typical fine structure of the *B. subtilis* spore is shown in Fig. 2. The spore consists of the core at the center of cell and several layers including proteinaceous spore coat, outer forespore membrane, cortex, germ cell wall,
and inner forespore membrane. The exosporium, as a loose balloon-like layer [54,55], is found at the outermost layer of some species including *B. cereus* and *B. anthracis* but not of *B. subtilis* with the exception of a strain isolated from the human gastrointestinal tract [53]. The function of the exosporium is not well understood because the exosporium is dispensable for most laboratory tests of spore resistance [56, 57, 58]. The particular adherence and hydrophobic properties were conferred by the exosporium [59, 60] suggesting the possibility that it is enhancing pathogenicity in natural environment.

**Figure 2.** Fine structure of *B. subtilis* spore. The upper panel shows a thin section electron micrograph of a dormant spore and the lower panel shows a magnified view of the spore layers.
The spore coat has two morphologically distinct layers: an electron-dense outer layer called the outer coat, and a less electron-dense inner layer with a lamellar appearance called the inner coat [61]. Proteinaceous coat layer contains at least 70 protein species in size from 6 to 70 kDa [62, 63]. Resistance of spore to harsh environmental conditions is due to both the complex structure of the shells and the unique physiological state within the core [64, 65].

The outer forespore membrane, located between coat and cortex, is essential for spore formation [66]. However, this membrane may not retain its integrity in dormant spore and consequently is likely not a significant permeability barrier. Indeed, removal of the outer membrane has no notable effect on spore resistance to heat, radiation and some chemicals [47, 67].

The cortex, beneath the outer forespore membrane, is composed by the part of peptidoglycan with a structure similar to cell wall of vegetative cell. The mesh structure of cortex contains strands of N-acetyl-glucosamine and N-acetyl-muramic acid residues and peptide chains, and approximately half of the muramic acid residues are converted to muramic-δ-lactam and only L-alanine [68]. The cortex keeps the spore core water activity relatively low, which is essential for the development and maintenance of resistance, but detailed mechanism is not clear yet. The germ cell wall is composed of peptidoglycan similar to cell wall of vegetative cell, and become the cell wall of the newly formed vegetative cell (outgrowing cell) during germination. The exact function of the germ cell wall is not well understood. Inner forespore membrane contains lipid similar to plasma membrane of growing cells [69, 70, 71] and is a strong permeability barrier that plays a major role in spore resistance to many chemicals [47, 70, 71].

Spore core is an equivalent part to a cytoplasm fraction of the vegetative cell. The core contains various enzymes and components that required in an energy metabolism and synthesis of macromolecules. A major factor in spore dormancy and resistance is considered as follows; (i) the low level of water content in the core, which are estimated about 28–57% of the mass of the hydrated dormant spore core as compared with the level of about 75 to 80% in the vegetative cells [62, 67, 72], (ii) the high level of pyridine-2, 6-dicarboxylic acid (dipicolinic acid, DPA) (~10% of the mass of the hydrated stage of spore core) in the spore core, likely present as a chelate with divalent cations, predominantly Ca²⁺[62, 73], and (iii) high level of small, acid-soluble spore proteins (SASPs). Very low level of water in spore core may limit macromolecular movement for enzymatic action and also keeps ions in immobile state that allows the core in a glass-like state [74], which might contribute for spore dormancy.
4. Unique components of spores

Spores contain several specific components, which are not found in vegetative cells including dipicolinic acid, spore photoproduct, small acid-soluble spore proteins, and spore coat proteins.

(1) DPA

Dipicolinic acid (DPA) (Fig. 3) is a unique chemical to bacterial spores and it comprises 5 to 15% of the dry weight of spores in many species of Bacilli and Clostridia. DPA is synthesized only in the mother cell compartment during sporulation and is taken up into the forespore [75] thus locates in the core probably as a chelated state with divalent cations, mainly Ca²⁺[72]. High level of DPA content makes a major contribution to heat resistance of spores, while the precise state of DPA in core is not well characterized. The accumulation of high level of DPA in the core is required for reduction of core water content during sporulation, and DPA also plays a significant role in protection of DNA in spores from UV radiation [73, 76]. DPA in the spore core is subsequently released within few minutes during germination.

![Figure 3. Structure of dipicolinic acid (DPA).](image)

(2) Spore photoproduct (SP)

Spores are 10- to 50-fold more resistant than growing cells against UV radiation of 254 nm [47]. In the vegetative cells, major products from DNA generated by UV radiation are cyclobutane dimmers (CPDs) and (6-4)-photoproducts (64PPs) where as predominant photoproduct generated in spore is a thymidyl-thymine adduct, 5-(α-thyminyl)-5,6-dihydrothymine, (called as spore photoproduct, SP) formed between adjacent pyrimidines in the same DNA strand (Fig. 4). The SP is much less hazardous than CPDs or 64PPs in the core, and it will be repaired in the first minutes of spore outgrowth by spore photoproduct lyase (SP lyase) [47]. SP lyase is produced during sporulation, packaged in the dormant spore, and activated early during germination then monomerize SP in situ back to two thymines [77, 78, 79]. The detailed property of SP lyase was described by Chandor-Proust et al [80].
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Figure 4. Structures of UV photoproducts formed in DNA in spore. Structures of (a) spore photoproduct (SP), (b) cyclobutane dimer, and (c) (6-4)-photoproduct are shown. These photoproducts are formed between adjacent thymidine residues on the same DNA strand.

(3) SASPs

Small, acid-soluble spore proteins (SASPs) are unique and predominant proteins of the core composing as much as 20% of the total spore proteins [81]. SASPs are low-molecular weight acid-soluble proteins with 60 to 75 amino acids. These proteins are mainly classified into three groups; α, β, and γ based on sequence comparison and biochemical properties [82]. The α/β-type SASPs is synthesized only in the developing forespore at late during sporulation, slightly prior to DPA uptake. These proteins are highly conserved only in the spore forming bacteria of Bacillus and Clostridium but not in non-spore formers, and it exhibit no structural motifs identified in other proteins. The α/β-type SASP deficient mutant spores of B. subtilis are
sensitive for a wide range of damaging agents, including heat, UV radiation, nucleases, dry treatment, hydroxyl radicals, nitrogen oxide, formaldehyde and desiccation [75] suggesting that SASPs play important roles in spore resistance. The α/β-type SASP binds to DNA and alters DNA conformation locally from a B to an A conformation [83]. γ-type and other types of SASP are also found in spores but the detailed function of these proteins is not clear yet.

(4) Spore coat proteins

The spore coat layer is essential for resistance of spore to various chemicals and to exogenous lytic enzymes. The coat is mainly formed of protein, and also contains some polysaccharide [84]. Approximately 70% of coat proteins can be solubilized from the purified spores by treating with a combination of reducing and denaturing agents at alkaline pH. The spore coat is a complex, multilayered assemblage of at least 70 protein species range in size approximately from 6 to 70 kDa. A typical example of the SDS-polyacrylamide gel electrophoresis (PAGE) profile of spore coat proteins extracted from wild type and mutant spores of B. subtilis are shown in Fig.5 [85].

Figure 5. SDS-PAGE analysis of spore coat proteins. Spore coat proteins from wild-type (A) and yabG mutant spores (B) of B. subtilis were solubilized by treatment with SDS and 2-mercaptoethanol, run on 13% acrylamide gels and stained with Coomassie Brilliant Blue. The arrowheads indicate proteins identified by N-terminal amino acid sequencing.
An investigation of spore coat has been developed in the following approaches; the first is an isolation of each coat components from the whole spore or purified coat fraction, the second is an analysis of biochemical nature of the each coat protein, and the last is studying the morphogenesis of complex coat structure. Many spore coat proteins have been identified by reverse genetics, following extraction and electrophoretic resolution of coat proteins, either by N-terminal sequence analysis or by LC/MS [45,85]. Although disruption of any one gene encoding a spore coat protein typically has little or no effect on spore resistance, morphology, or germination, a few proteins have been found to be critical for spore coat assembly referred to as morphogenetic proteins. These proteins include CotE, SafA (YrbA), SpoIVA, SpoVM, and SpoVID [44, 63]. SpoIVA is needed for directing assembly of the coat to its proper location around the outer surface of the developing spore [86]. CotE also has a primary role in spore coat formation and is responsible for the assembly of outer spore coat [87]. In addition to these two proteins, SpoVM, SpoVID, and SafA are also important for coat morphology. Morphogenesis of coat layer begins just after formation of sporulation septum (Fig. 1b) but each coat proteins are synthesized at well-defined stages of sporulation in mother cell until maturation of spore (Fig. 1b-f).

5. Morphogenetic proteins in spore coat formation

Spore coat proteins are produced in the mother cell compartment and assemble around the forespore [88]. Expressions of these genes are temporally regulated by the transcription factors $\sigma^E$, SpoIIID, $\sigma^K$ and GerE in the mother cell (Fig. 6B). Expression of genes encoding proteins required at early stage of spore coat formation is directed by $\sigma^E$ and then SpoIIID activates genes required at little later. After the completion of engulfment, $\sigma^K$ directs genes for spore coat formation and approximately an hour later, the small transcription factor GerE works with $\sigma^K$ to direct expression of additional coat protein during the latest stage of sporulation [93].

During the formation of spore coat structure, the first morphogenetic protein SpoIVA (55-kDa), encoded by spoIVA gene, is expressed under the control of the mother cell specific $\sigma$ factor $\sigma^E$ from 2h after the onset of sporulation. SpoIVA is located on the mother cell side of the polar septum (Fig. 1b and c) and outer forespore membrane (Fig. 1d) then attaches the matrix to the forespore [91, 95,96]. Mutation in spoIVA results in an unusual defect of spore coat in which the spore coat misassembles as swirls within the mother cell cytoplasm are observed[86,97]. The cortex layer is also defective in the mutant spores, indicating that SpoIVA is necessary for proper
formation of both cortex and spore coat [97,98]. Localization of SpoIVA requires a σE-controlled small peptide SpoVM and at least one other unidentified factor [96]. C-terminal 5 amino acid residues of totally 492 amino acid and at least the region centered at position 393 in SpoIVA protein are essential for its targeting to the developing spore [96,99]. Recently, Ramamurthi and co-workers demonstrated that SpoIVA possesses a canonical Walker A box that binds and hydrolyzes ATP, which is needed for multimerization and assembly of SpoIVA into filamentous structures forming a shell encasing the forespore [100].

SpoVID (575 amino acids coat protein, encoded by spoVID gene) is involved in the attachment of CotE to the forespore. SpoVID contains a cell wall-binding LysM motif in its amino acid sequence, facilitating its assembly to the cortex-coat boundary [101,102]. The same motif was found in the N-terminal part of another coat protein, SafA [103]. Ozin and co-workers have discovered that SafA (previously known as YrbA) forms a complex with SpoVID [101]. SafA is required for normal outer coat formation, germination, lysozyme resistance of spore, and the assembly of several spore coat proteins including CotG [104]. SafA and SpoVID proteins interacted directly [105]. This interaction is mediated by two motifs present in SafA sequence, the PYYH motif in the C-terminal half and a region just downstream of the LysM domain consists of 13 residues. Müllerová and co-workers observed direct interaction between SpoIVA and SpoVID proteins using two-hybrid assay and verified by coexpression experiment followed by Western blot analysis, and also revealed a novel interaction between SpoIVA and SafA [106].

Another morphogenetic protein CotE, a 24 kDa of an alkali-soluble coat protein, is a prerequisite for the localization of SpoIVA close to the outer forespore membrane [88, 91]. CotE located at the boundary of the inner and outer coat layers as demonstrated by immunoelectron microscopy [91]. CotE forms a layer with a small distance on the layer of SpoIVA at the septum, and the space between SpoIVA and CotE is apparently filled by material of unknown composition called matrix [96]. Although CotA and CotC require CotE to localize on spore, the expressions of the cotA and cotC genes were unimpaired in cotE mutant cells, thus CotE is involved in the assembly of CotA and CotC on spores but not in the expressions [87, 94]. Ultrastructural analysis indicated that the cotE deficient mutant spore lacked the electron-dense outer layer of spore coat but retained a normal-looking inner coat. Therefore, most or all outer coat proteins require CotE to assemble on spore. Our results showed that the incorporation of CotS, which located in the inner coat layer, is also dependent on cotE [92,107]. CotH, which is needed for proper formation of both inner and outer spore coat, required CotE for the
assembly [108]. These results suggest that CotE is involved in the assembly of most or all outer coat proteins and some inner coat proteins.

6. Spore crust

Immunoelectron microscopy is a conventional method used to determine the localization of spore coat proteins. The location of CotE, CotS, and SpoIVA in the spore coat was determined by immunoelectron microscopy (Fig. 6A italicized proteins) [91, 92]. However, this method requires special training and equipment and thus sometime results are opaque. Therefore, for most of the spore coat proteins, their position in the coat has not been determined experimentally, although provisional assignments were made based largely on control of assembly into the coat by CotE [94]. One of the morphogenetic proteins, CotE, is located between the inner and outer coats, and directs assembly of most or all of the outer coat proteins and also a few of the inner coat proteins [88, 91, 94, 109, 110]. Therefore, spore coat proteins located on spore without CotE were assigned to inner coat and proteins that require CotE to localize on spore were assigned to outer coat. However, these provisional assignments are not definitive because some inner coat proteins are CotE dependent and proteins in inner and outer coat are dependent on each other [94]. Recently, we developed a new useful method to determine the location site of coat proteins in which diameter of protein layer was compared to the diameter of the corresponding spore using fluorescence microscopy [89] (Fig. 7). Our results suggested that CotD, CotF, CotT, GerQ, YaaH, YeeK, YmaG, YsnD, and YxeE are present in the inner coat and that CotA, CotB, CotC, and YtxO reside in the outer coat. In addition, CotZ and CgeA appeared in the outermost layer of the spore coat [89]. McKenney et al also developed similar method and locations of totally 27 proteins were determined [90] (Fig. 6A). These methods revealed the previously unidentified outermost spore layer named crust (Fig. 1f, Fig. 6A). Some of these assignments did not fully agree with the assignments by immunoelectron microscopy (Fig. 6A). However, we note that our study and the work by McKenney et al were performed independently and 6 proteins were analyzed in both work but there were no disagreement in assigning proteins into inner coat, outer coat and crust [89, 90]. Crust was externally exposed outermost layer of *B. subtilis* spore and it covers spore coat [111]. We also demonstrated that CotY is an additional component of crust and CotY and CotZ play critical role in crust formation [111] (Fig. 6A). Fig. 6B shows a summary of expression control of spore proteins based on the previous review [93]. The expressions of inner coat proteins were controlled by transcription factor $\sigma^K$ (and GerE) or $\sigma^E$ (and SpoIII)D. Most outer coat
Figure 6. Location of spore proteins and control of their expression. (A) Location site of spore proteins [89, 90]. Proteins in italic are determined by immunoelectron microscopy by Driks et al (CotE and SpoIVA) [91] and Takamatsu et al (CotS) [92]. (B) Control of expression of spore coat and crust proteins by mother cell specific transcription factors are summarized referring to the reviews by Driks [93] and Takamatsu et al [63]. Proteins in boldface type are known to affect coat structure or biochemical composition. *: Proteins, which are known to require CotE for the assembly on spore [94, 89, and unpublished data].

proteins were controlled by $\sigma^K$ and no $\sigma^E$-controlled protein was assigned to outer coat except for CotE among 28 proteins in Fig. 6A. All crust proteins required $\sigma^K$ and GerE for the expression. These observations suggest the tendency that proteins in outer layer are produced later during sporulation than proteins in inner layer. On the other hand, expressions of some inner coat proteins were directed by $\sigma^K$, suggesting that the inner coat is accessible to these proteins until late during sporulation. Our investigation demonstrated that CotZ and CgeA appeared to be more abundant at the mother cell proximal pole of the forespore, whereas CotA and CotC were more abundant at the mother cell distal pole of the forespore [89]. Moreover,
Figure 7. Localization of proteins to different layers of *B. subtilis* spore. Points indicate the average localization sites of each protein and error bars indicate standard deviations of results for 60 spores [89].

CotB was observed at the middle of the spore as a ring-or spiral-like structure. Formation of this structure required *cotG* expression. These observations suggest that spore coat assembly is more intricate than previously appreciated.

7. Overview of spore survival

Bacterial spores are highly dormant and resistant to several harsh environments for over decades, centuries, or sometimes several million years. There are multiple mechanisms for resistance of bacterial spores against various stresses as reviewed in this chapter. Major factors involved in spore dormancy are spore coat, cortex, inner membrane, dehydrated state of the
core, saturation of spore DNA by α/β-type SASP, DPA accumulation into the core, and DNA repair system. The spore coat provides a barrier against lytic enzymes, toxic chemicals and UV radiation. The low permeability of inner forespore membrane retards the influx of toxic chemicals into the core. Cortex is responsible for maintaining the highly dehydrated state of the core, thereby contributing to the extreme dormancy and resistance to wet heat and γ-radiation of spores. The α/β-type SASP saturation of DNA would protect it against wet and dry heat and some genotoxic chemicals. DPA accumulation in the core affects to hydration of the core and mineral levels as well as wet heat and UV resistance of spores. DNA repair system is important for resistance of spores to DNA damage by radiation or genotoxic chemicals. These multiple systems ensure dormancy, resistance, and survival of spores and thus bacterial spores became one of the longest living organisms in the world.

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